THE UNITED STATES PATENT AND TRADEMARK OFFICE

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For: 8-HYDROXY QUINOLINE DERIVATIVES

Confirmation No.: 7111

Commissioner for Patents
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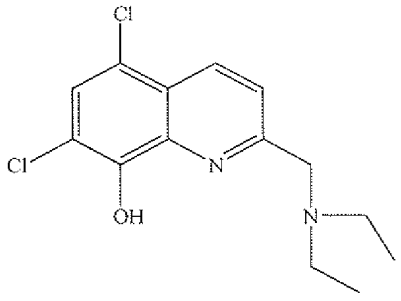
SUPPLEMENTAL DECLARATION OF DR. ROBERT CHERNY

Sir:

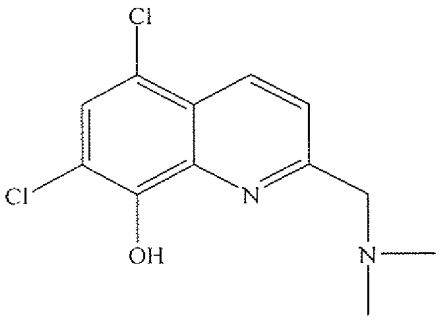
I, Robert Cherny, hereby declare and state as follows:

1. This Declaration is Supplemental to my earlier Declaration dated September 16, 2008, in the above-identified case.

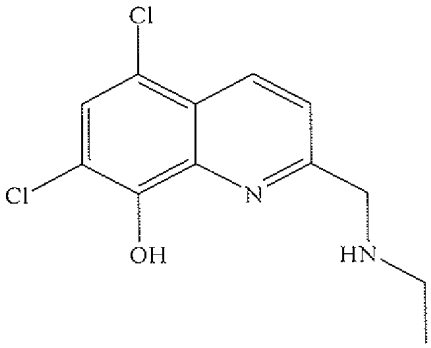
2. The ultimate objective of the experiments described in this Declaration is to compare the ionophore properties of the compound disclosed in U.S. Patent No. 3,682,197 of Carissimi et al. ("Carissimi et al.") having the structure:



(designated as "CC") with compounds of the present application having the structures:



PBT 1033



PBT 1051

(designated as "1033" and "1051", respectively).

3. This Declaration is provided to present all of the ionophore assay data which has become available to the declarant to date that was conducted in a comparison with respect to a compound of the present invention and CC. This Declaration also confirms and reiterates the conclusions rendered in the previous Declaration.

4. The compounds above were tested for their ionophore properties in accordance with the following procedure (referred to as the "ionophore assay") under my direct supervision and control. This assay is the same as described in my previous Declaration.

5. M17 human neuroblastoma cells were plated out on 6 well tissue culture plates and left overnight. Sufficient cells were provided to achieve approximately 70 % confluence (1 million cells). Cells were incubated in a media of 1 ml of Opti-MEM (Invitrogen) with added 10% FBS, Sodium Pyruvate, NEAA, and PenStrep and 10 μM of $CuCl_2$ for 5 hr at 37°C. The cells were also incubated with or without the various test compounds (CC, 1033 or 1051 or CQ for a positive control). 2 or 10 μM concentrations of each of these test compounds, as indicated below, were added to each set of cultured cells. At the conclusion of the incubation, the media were removed using a vacuum aspirator, and 1 ml of PBS was added to dislodge the cells. Cells

were then transferred to microfuge tubes and pelleted. The PBS was discarded and the remaining cell pellets were frozen at -20°C. The cell pellets were assayed for metal content using inductively coupled plasma mass spectrometry (ICPMS) analysis.

The cell pellets were prepared as follows:

50 µl of concentrated Nitric Acid (Aristar, BDH) was added to each cell pellet, and the samples were allowed to digest overnight. Following this procedure, the samples were heated for 20 min at 90°C to complete the digestion. The volume of each sample was reduced to ~45 µl after digestion, to which a further 1 ml of 1% Nitric Acid diluent was added.

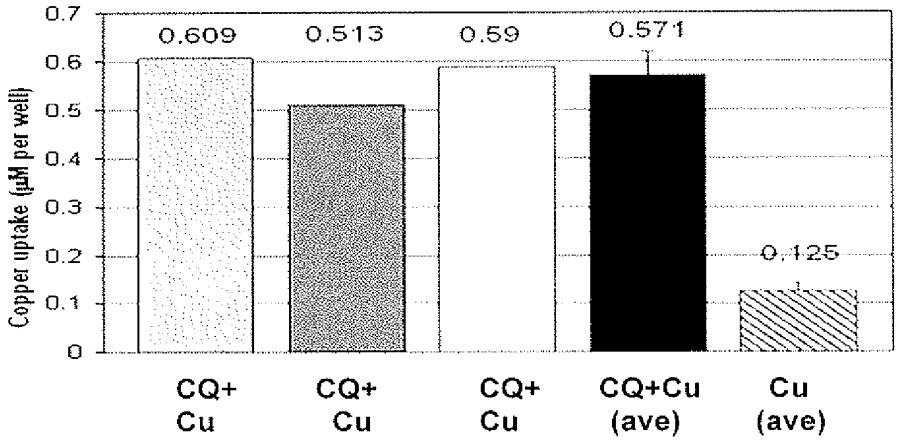
Measurements of the metal content of the neuroblastoma cells were performed using a Varian UltraMass ICPMS instrument under operating conditions suitable for routine multi-element analysis.

6. The ionophore assay was designed to assess the ability of compounds to promote the uptake of copper relative to the known copper ionophore, clioquinol (CQ). CQ is acknowledged in the literature as possessing positive ionophore properties and being capable of reducing the levels of Abeta, a common target protein of Alzheimer's disease therapies. I included CQ as the positive control in all the assays and internally ranked the drug candidate compounds as relative to CQ. This assay was used to obtain values with respect to 1033, 1051 and CC which were compared to CQ as a positive control compound.

7. As stated above, the present Declaration is provided to present all of the ionophore assay data of which I am aware, which compares the compounds of interest, namely 1051 and/or 1033 and CC, identified above in Paragraph 2. Compared to the positive control CQ, the data clearly evidence that 1051, and especially 1033, exhibit enhanced ionophore properties.

8. In order to establish the consistent ionophore performance of CQ and its use as a positive control compound, the results for this compound over different assays are grouped below in Graph A for comparison. CQ at a concentration of 10 μM was tested using the ionophore assay described above.

Graph A



9. Each of the experiments depicted in Graph A was conducted on different dates. As shown by the graph, the amount of copper uptake in cells exposed to 10 μM copper in each of the three assays conducted in the presence of 10 μM CQ was 0.609, 0.513 and 0.59 μM, providing an average uptake of 0.571 μM. The average amount of copper uptake in the absence of any drug (Cu average) was 0.125 μM. Accordingly, the values for CQ as a control at 10μM were observed to be very consistent and readily reproducible. From my experience, CQ has been found to provide a stable benchmark, reliably promoting metal uptake relative to the quantity of copper taken up by M17 neuronal cells which had not been exposed to any drug.

10. When 1033 was initially studied in this assay at 10μM and on several occasions subsequently, I found it to reproducibly provide very high readings relative to CQ. However,

those readings with respect to 1033 varied from assay to assay within the high reading range. This same phenomenon was observed with other compounds that are extreme ionophores, leading me to conclude that the assay readout was not particularly sensitive to distinguish variations within a very high reading range where these potent compounds were concerned.

11. I considered two explanations for the above observation:

(a) At concentrations considered high for potent compounds (10μM) the cells were taking up so much copper so rapidly that small variations in assay conditions (e.g., the placement of the wells, time taken to load the culture plates, etc.) might be leading to intra-assay variation.

(b) At high concentrations, the rapid uptake of copper may have variable effects upon cell biology which might lead to saturation or toxicity effects on the cells.

12. As a result, I tested the compounds which were found to be very potent ionophores (300% or more relative to CQ) at a concentration which brought them into the reading range of CQ and similar moderate ionophores. At 2 μM, 1033 was generally found to generate a reading in the range of 100% of CQ at 10 μM. In the early experiments (discussed above) and prior to observing variations in its performance, 1033 was tested at 10 μM until I decided to change its concentration to 2 μM. Each experiment discussed below identifies the concentration of 1033 utilized.

13. Assays were subsequently conducted on compounds 1033, 1051, CQ, and CC. All the compounds in these assays were compared to CQ and Cu alone (in the absence of test compound). The results of these assays and experiments 1, 2 and 3 are summarized in Table A below, which includes reference to Graphs B and C, which are explained later in this declaration:

Table A

EXPERIMENT NUMBER	**CONTROLS**		**TEST COMPOUNDS**		
	Cu alone	**CQ** + Cu	**1033** +Cu	**1051** +Cu	**CC**+ Cu
Experiment 1 (Graph B)	0.11	0.61 (10μM)	2.73 (10μM)	1.65(10μM)	
Experiment 2 (Graph C)	0.11	0.59(10μM)	0.78 (2μM)		1.18(10μM)
Experiment 3	0.14	0.513(10μM)	1.51 (2μM)	1.74(10μM)	1.32(10μM)

Note that in Experiments 2 and 3, 1033 was utilized at the 2 μM level.

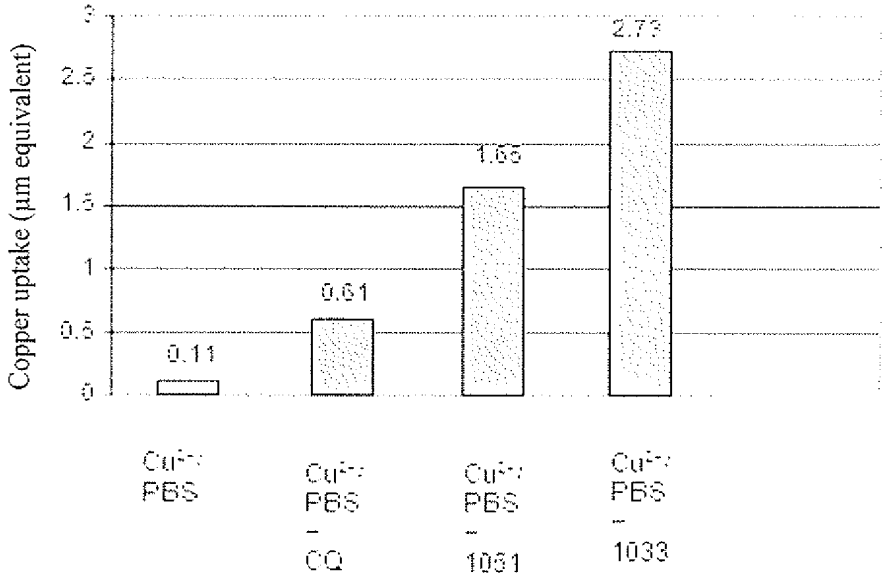
These experiments are described in more detail in the following paragraphs.

14. In Experiment 1, the amount of copper uptake for 1033 and 1051 was compared in the ionophore assay, where all of the drugs were tested at 10 μM. (This experiment was conducted prior to my decision to measure the ionophore effect of 1033 at 2 μM.) The results of Experiment 1 are depicted in Graph B below:

Graph B

Results of Experiment 1

INITIAL COMPARISON OF COPPER UPTAKE OF 1051 AND 1033 RELATIVE TO CQ



15. In Graph B and in the graphs below, it is to be understood that the term "equivalent", as used on the vertical axis, indicates that the results have been averaged over more than one assay.

16. The amount of copper uptake when CQ was present was 0.61 µM, and the value of copper uptake in the absence of any test compound (control) was 0.11 µM . The amount of copper uptake in the presence of 1033 was 2.73 µM, while the amount of copper uptake in the presence of 1051 was 1.65 µM. Notably, this assay did not include CC.

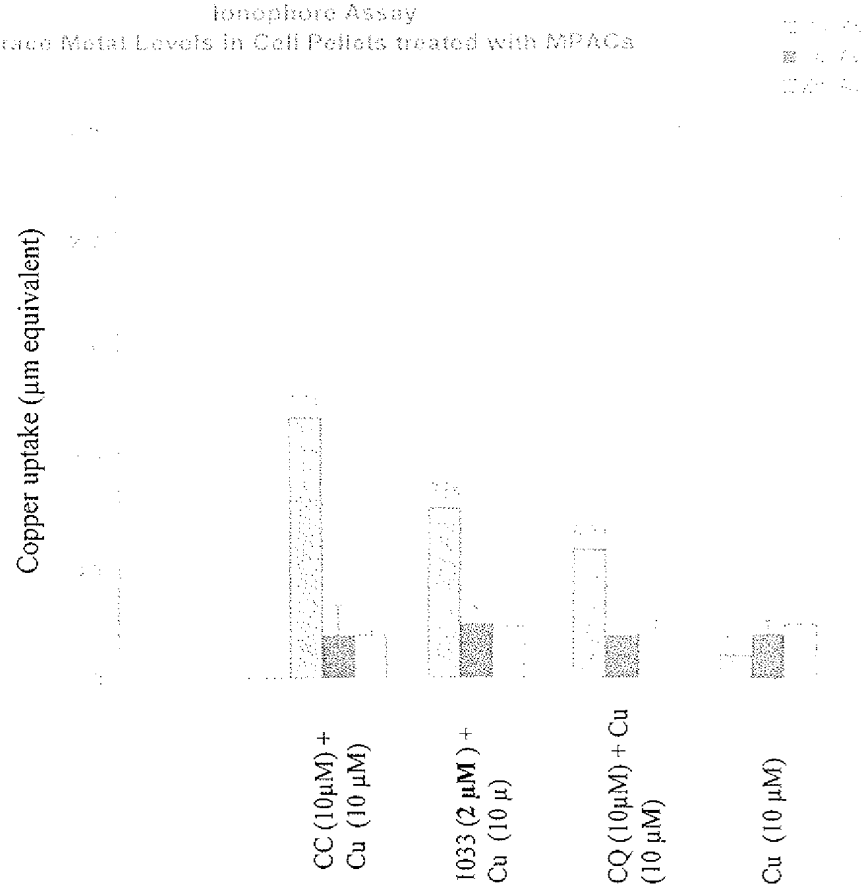
17. In Experiment 2, I compared the amount of copper taken up in the absence of any drug with copper taken up in the presence of CC, 1033 and CQ, in accordance with the protocol described. The protocol was performed with 1033 at 2 µM concentration for the reasons provided

in Paragraphs 9-12 above. (The graph also depicts the amount of iron and zinc uptake, but these data are irrelevant to the discussion herein.) Experiment 2 was the first time that my laboratory conducted the ionophore test with CC. The results of Experiment 2 are depicted in Graph C below:

Graph C

Results of Experiment 2

COMPARISON OF COPPER UPTAKE OF CC TO CQ



Notably, as shown in Graph C, the average values for copper uptake in the presence of CQ and in the absence of any test compound in Experiment 2 was 0.59 µM and 0.11 µM, respectively. In fact, the value of copper-uptake in Graph B (INITIAL COMPARISION OF

COPPER UPTAKE OF 1051 & 1033 RELATIVE TO CQ) and Graph A (COMPARISON OF COPPER UPTAKE OF CC TO CQ) were exactly the same when no drug was present, i.e., 0.11 μM.

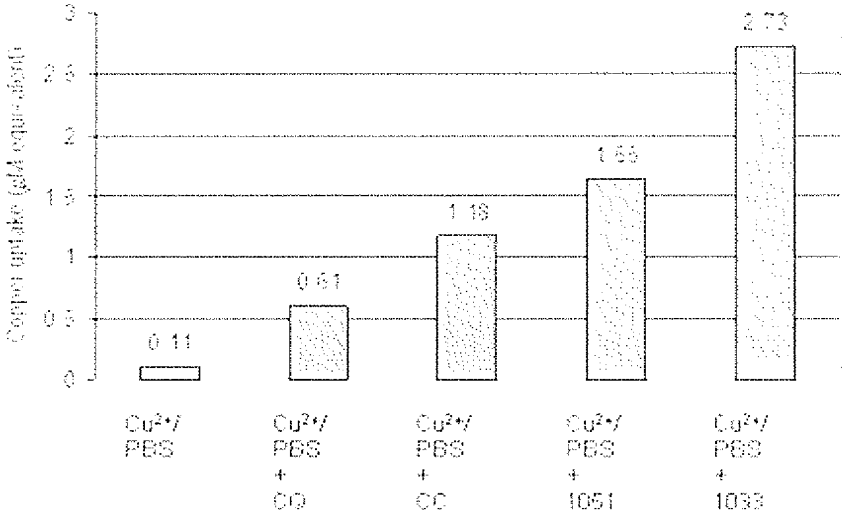
18. Since the values obtained for both the positive control (CQ) and negative control (metal alone) were consistent and reproducible at such low concentrations in the ionophore test, I was entirely confident that the values relating to the amount of copper uptake by compounds 1051, 1033 and CC in the ionophore test, conducted according to the assay described above, would also be consistent and reproducible, whether CC were tested side by side with the other test compounds or at a different time to 1051 and 1033.

19. Accordingly, although Experiments 1 and 2 were conducted several months apart, I felt it was appropriate and justifiable to include the data obtained from the assay of CC conducted in Experiment 2 (COMPARISON OF COPPER UPTAKE OF CC TO CQ) with the data obtained in Experiment 1 (INITIAL COMPARISION OF COPPER UPTAKE OF 1051 AND 1033 RELATIVE TO CQ). In other words, I took the values for the copper uptake when 10 μM of CQ, 1051 and 1033 were present, generated in Experiment 1, and the value for copper uptake in the presence of 10 μM CC obtained from the data generated in Experiment 2 and combined these data, as represented in bar graph D, which are the same data which I presented in my earlier Declaration dated September 16, 2008. It is my opinion that the results depicted in Graph D clearly show that 1051, and especially 1033, exhibited enhanced ionophore performance relative to CQ, the positive control, as well as relative to CC. Since the assay protocol is the same for all compounds presented, and since, as described above, the data with respect to the amount of copper uptake in the presence of CQ or in the absence of any drug, were reproducible, it is inconsequential that the data obtained with respect to CC were generated at

different times than the data generated for 1051, 1033, CQ and the control, for the purpose of the results shown. These data are reproduced below in Graph D:

Graph D

COMPOSITE OF GRAPHS B & C



20. The control experiment in the absence of drug (Cu/PBS) indicated that there was some uptake of copper by the cells. As shown by the data depicted in Graph D, when CQ was added, the amount of copper taken up was 0.61 μM; for CC, the amount of copper taken up was 1.18 μM; for 1051, the amount of copper taken up was 1.65 μM; and for 1033, the amount of copper taken up was 2.73 μM.

21. From Graph D, it can be seen that when present at 10 μm, 1033 exhibited about a 4.5 fold enhancement in ionophore performance relative to CQ, while 1051 exhibited about a 2.7 fold enhancement in ionophore performance. CC exhibited an increase of about 1.9 fold in ionophore performance relative to CQ.

22. The results of Experiment 3, as detailed in Table A, were performed after

experiments 1 and 2 and confirm my earlier conclusions. Experiment 3 involved CC, 1033 and 1051 and the control conducted in a side-by-side comparison at the same time in accordance with the protocol for the ionophore assay described herein. Notably, as indicated above, since 1033 is a powerful ionophore, the concentration of 1033 utilized in this assay was 2 μM.

23. Again, the amount of copper uptake at 10 μM for CQ was 0.51 μM. Further, when no test substance was present, the amount of copper uptake was 0.14 μM. The value for copper uptake in the presence of CC was 1.32 μM, while the value for copper uptake in the presence of 10 μM of 1051 was 1.74 μM. The value of copper uptake in the presence of 2 μM 1033 (one fifth of the concentration of CC) was 1.51 μM, which when extrapolated to 10 μM, would be significantly higher than the value reported in Graph B (INITIAL COMPARISON OF COPPER UPTAKE OF 1051, 1033 RELATIVE TO CQ (Experiment 1)) and Graph D (which is identical to the bar graph shown in paragraph 19 of my earlier Declaration dated September 16, 2008). As described above, the lower uptake number of 2.73 μM for 1033 in Graphs B and D was attributable to saturation effects.

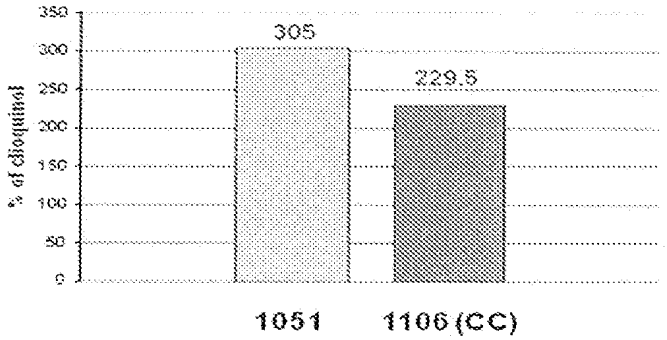
24. The data in Graph D and Experiment 3 confirm that 1033 is a far superior ionophore to CC and, in my opinion, would be expected to provide a significantly enhanced capability relative to CC in reducing the levels of Abeta, a common target of Alzheimer's disease therapies. Further, the data in Graph D and Experiment 3 confirm that 1051 is clearly a superior ionophore to CC. This conclusion is further shown by the data in Graph E, described in Paragraph 26.

25. For purposes of comparison, 1051 and CC were averaged over all assays and normalized against the positive control compound clioquinol (CQ) on the two occasions in which these compounds were tested. In this comparison, compound 1051 evidenced a superior

ionophore capability than CC, with 1051 having an ionophore performance 33% greater than CC, as shown in Graph E below:

Graph E

COMPOUNDS NORMALISED AGAINST POSITIVE CONTROL COMPOUND (CLIOQUINOL)



26. Based on all of the data generated, it is my opinion that 1051 is a superior ionophore to the CC and that compound 1033 is a far superior ionophore to CC. As such, both compounds are expected to be significantly more effective in reducing the levels of Abeta.

27. I declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that those statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

By: _____ Dated: May 7th 2009

Robert Cherny